

3-AMINOPROPYL-BONDED-PHASE SILICA, THIN-LAYER CHROMATOGRAPHIC PLATES: THEIR PREPARATION, AND APPLICATION TO SUGAR RESOLUTION

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ABSTRACT

Commercially available, precoated plates of silica gel have been derivatized by reaction with (3-aminopropyl)triethoxysilane (3-APTS). The aminopropyl-bonded-phase plates prepared from Adsorbosil-Plus-1, soft-layer prekotes (Alltech Associates, Inc.***) provided optimal resolution of a variety of closely related sugars. Plates were immersed in a 1.0% solution of 3-APTS in hexane for 15 min, washed with hexane, and then dried. The resulting, bonded-phase silica contained 9.7% of 3-aminopropyl groups. Further derivatization could be obtained, but optimal resolution of sugars occurred at this level of aminopropyl substitution. Acetonitrile-water mixtures were used as mobile phases; normal phase chromatography resulted, because increasing the proportion of water resulted in increased mobility of the sugars. Closely related mono-, di-, and tri-saccharides have been resolved more effectively than in such traditional approaches as t.l.c. on plates of cellulose or underivatized silica. Also, significantly less time is required for irrigation of the plates than is normally encountered in the t.l.c. of sugars. "Anomalous" behavior was observed for aldotetroses, aldopentoses, and some aldohexoses and deoxy sugars; these remain at the origin. Two sugars of this series, xylose and rhamnose, have been shown to interact covalently with the amino groups on the derivatized plates.

INTRODUCTION

For liquid chromatography (l.c.) under elevated pressure (~10 MPa), columns based on silica gel modified with bound 3-aminopropyl groups have been commercially available for several years. The columns have been used extensively for sugar separations, and are very effective. Several reports¹⁻⁴ described the prep-

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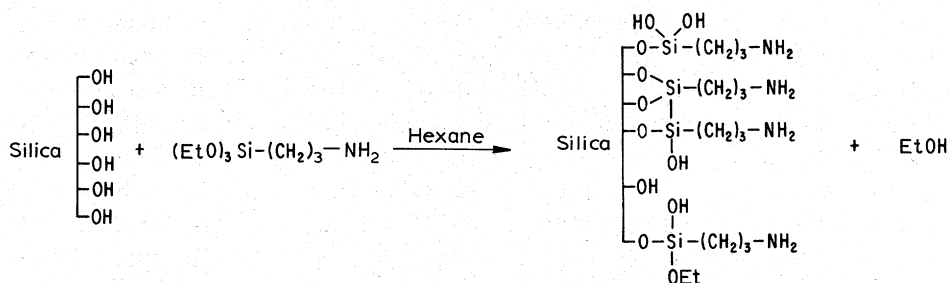
***Reference to a brand or firm name does not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

aration of such columns after derivatization of silica by reaction with (3-aminopropyl)triethoxysilane (3-APTS). In these studies, the bonded-phase packing-materials were prepared by stirring silica gel in solutions of 3-APTS in such nonpolar, organic solvents as hexane, benzene, or toluene.

More recently, efforts have been directed⁵⁻⁹ toward extending these procedures to the preparation of (aminopropyl)silica plates for t.l.c. Some researchers^{5,6} have separated metals on plates of silica that had been derivatized by reaction with 3-APTS. Others^{8,9} have successfully resolved D-fructose, D-glucose, and lactose by using plates precoated with silica and derivatized by immersion in a solution of 3-APTS in hexane. As described, their procedure for plate derivatization was very lengthy, requiring several days for reacting, washing, and drying the plates. We have decreased this time to 1 h, and have enhanced the resolution of the sugars. Separations achieved are, in most cases, superior to those obtained by the traditional approaches¹⁰ to t.l.c. of sugars.

RESULTS AND DISCUSSION

3-Aminopropyl-bonded-phase, t.l.c. plates were prepared from plates precoated with silica gel that were supplied by several commercial vendors; we found that those from one source (see Experimental section) yielded bonded-phase plates that provided superior resolution of closely related sugars. These soft-layer plates (250- μm layer thickness, with 10% of CaSO_4 binder) are described as having been prepared from silica particles ranging in size from 6 to 13 μm , optimal for irrigation time and efficiency of separation. Although plates from other sources were readily derivatized, sugars generally displayed much lower mobilities when the same



Scheme 1. Reaction scheme for the derivatization of surface silanols in silica gel by reaction with (3-aminopropyl)triethoxysilane.

acetonitrile–water, mobile phase was used. The scheme for derivatization of surface silanol groups in silica gel with 3-APTS is depicted in Scheme 1. Derivatization can take several forms, and the degree of cross-linking increases with the moisture content of the plates⁶.

Preparation of 3-aminopropyl-bonded-phase, t.l.c. plates. — We found it un-

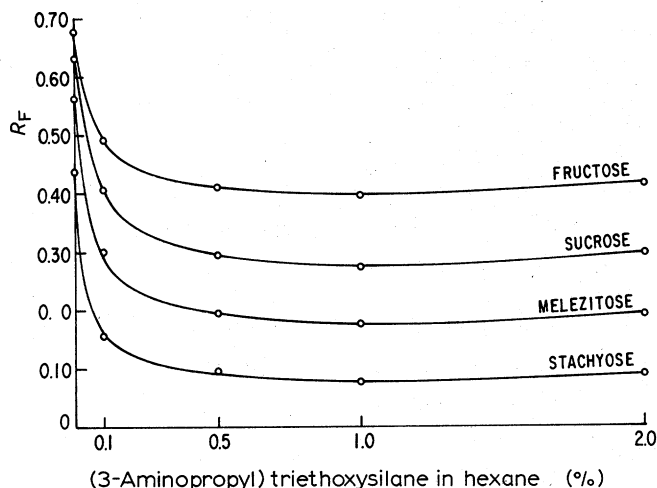


Fig. 1. Effect of the percentage of 3-APTS in hexane on the R_F values of sugars on the resulting plates. (Plates were immersed for 30 min.)

necessary to ensure that the plate was moisture-free prior to reaction with 3-APTS. Such procedures as drying the plates *in vacuo*, or over saturated solutions of LiCl, prior to derivatization, yielded bonded-phase plates that performed in the same way as those used directly from the container. Also, it was essential that the CaSO_4 binder be present in the precoated plates; those having no binder were found to resolve sugars very poorly.

Preliminary results showed that hexane and toluene perform equally well as the medium for the reaction of 3-APTS with silica t.l.c. plates, and therefore hexane was then used in all experiments. Various approaches were taken to prepare 3-aminopropyl-bonded-phase plates that would resolve sugars with maximum efficiency. Clearly, immersing plates in solutions of 3-APTS is the most effective approach. Attempts at preparing "gradient" plates by irrigation with solutions of 3-APTS in hexane were not successful; the silica near the origin became heavily derivatized with aminopropyl groups, and so sugars displayed minimal mobility.

Fig. 1 shows the effect of the concentration of 3-APTS in the immersing solution on the R_F values of D-fructose, sucrose, melezitose, and stachyose, using plates immersed for 30 min. The separation of the sugars was accomplished even after brief immersion in a 0.1% solution of 3-APTS in hexane. R_F values are further lowered from those on the uncoated plate by increasing the concentration of 3-APTS up to the 1.0% level, where they begin to increase slightly. Mobility of sugars on plates immersed in 10% 3-APTS in hexane have R_F values only minimally greater than those prepared in 2% 3-APTS. As reported previously, in the preparation of aminopropyl packing for l.c.², neither the time of reaction with 3-APTS nor the temperature has much effect on the degree of derivatization of silica, as the reaction proceeds very readily.

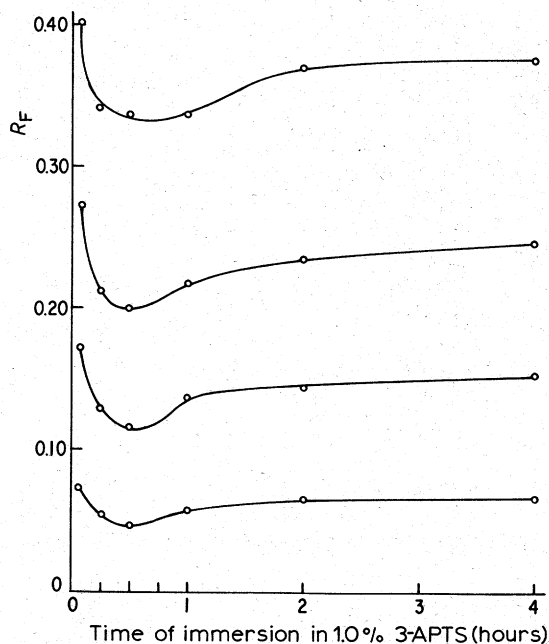


Fig. 2. Effect of time of immersion in 1.0% 3-APTS in hexane on the R_F values of sugars on the resulting plates.

The effect of the length of time of immersion of the plate in 1.0% 3-APTS in hexane on the resulting R_F values is shown in Fig. 2. Reasons for decrease in the R_F value over 30 min, and then a gradual increase, are unclear; it may result from the further derivatization with 3-APTS of amino groups in the initially formed (3-aminopropyl)silica, or from more cross-linking. From the results in Figs. 1 and 2, the procedure adopted for plate derivatization was a 15-min immersion in a 1.0% solution of 3-APTS in hexane. The spots derived from sugars upon charring with sulfuric acid spray were most compact, and the R_F values most suitable, when plates were derivatized in this way.

The acidic spray-reagent used here for detecting sugars was 6:4:3 *tert*-butyl alcohol-ethanol-sulfuric acid. Aminopropyl groups on the bonded-phase plates interfered with color formation after spraying with such traditional reagents as ammoniacal silver nitrate, periodate-benzidine, or diphenylamine-aniline¹⁰. The charring procedure used here detected all sugars, and the aminopropyl-bonded phase was not charred and provided a light background. Increased derivatization of the silica with aminopropyl groups (longer immersion time or higher % of 3-APTS in hexane) decreases the ease of sugar charring, constituting yet a further reason for using the 15-min immersion-time.

Samples of aminopropyl-bonded-phase silica were analyzed for carbon, nitrogen, and ash. From the results, both the degree of derivatization of the silica and the C:N molar ratio were calculated, and these are given in Table I. Bonded-phase

plates containing <10% aminopropyl substitution (15-min immersion) provided the most compact and detectable spots. Based on the nitrogen analysis, the aminopropyl content of the derivatized plates is ~1.0 mmol per g of derivatized silica. The presence of 10% of CaSO₄ binder in the silica was accounted for in determining the degree of derivatization of the silica. Reasons for the greater than theoretical ratio of carbon to nitrogen are not clear; perhaps a small proportion of ethoxysilane is retained (see Scheme 1).

By using a t.l.c. tank (20 × 20 cm) containing 1.0% of 3-APTS in hexane, as many as forty-eight 5 × 20-cm or twelve 20 × 20-cm bonded-phase plates have been prepared. This was accomplished by completely immersing eight small, or two large, precoated plates at one time, and, after their removal, adding the next batch. All of the plates performed similarly, as only negligible amounts of 3-APTS are removed by each successive immersion.

TABLE I

MICROANALYTICAL DETERMINATIONS OF 3-AMINOPROPYL-BONDED-PHASE T.L.C. PLATES

<i>Derivatization time (min)^a</i>	<i>Ash (%)</i>	<i>Bonded-phase (%)</i>	<i>Carbon (%)</i>	<i>Nitrogen (%)</i>	<i>Molar ratio of C:N</i>	<i>Theoretical C:N</i>
15	90.32	9.68	3.89	1.39	3.27	3.00
60	87.18	12.82	5.66	2.09	3.15	3.00

^aPrecoated plates of silica gel were immersed in a 1.0% solution of (3-aminopropyl)triethoxysilane in hexane, rinsed, and dried.

TABLE II

R_F VALUES OF SUGARS ON 3-AMINOPROPYL-BONDED-PHASE SILICA IN 7:3 ACETONITRILE-WATER

<i>Sugar</i>	<i>R_F</i>	<i>Sugar</i>	<i>R_F</i>	<i>Sugar</i>	<i>R_F</i>
<i>Monosaccharides</i>		<i>Disaccharides</i>		<i>Higher Saccharides</i>	
Sorbose	0.402	Sucrose	0.263	Raffinose	0.135
Fructose	0.406	Lactose	0.187	Melezitose	0.172
Tagatose	0.425	Lactulose	0.215	Maltotriose	0.119
Sedoheptulose	0.461	Cellobiose	0.200	Stachyose	0.064
Psicose	0.466	Cellobiulose	0.230		
Glucose	0.352	Maltose	0.200		
		Maltulose	0.220		
<i>Derivatives</i>		Turanose	0.238		
Methyl β-L-arabinopyranoside	0.636	Melibiose	0.165		
Methyl α-D-mannopyranoside	0.574	Gentiobiose	0.172		
Methyl β-D-glucopyranoside	0.540	α,α-Trehalose	0.210		
1,2-O-Isopropylidene-α-D-glucofuranose	0.657	Laminarabiose	0.233		
2-Acetamido-2-deoxy-D-glucose	0.404	Palatinose	0.235		
		Kojibiose	0.202		
		Isomaltose	0.215		

Evaluation of aminopropyl-bonded-phase t.l.c. plates. — The aminopropyl-bonded-phase t.l.c. plates exhibit normal phase characteristics, as increasing the ratio of water to acetonitrile in the solvent system results in increased R_F values. Not surprisingly, some fractionation of the components in the mobile phase occurs during chromatography; apparently, only acetonitrile moves to the front, and when 7:3 acetonitrile–water is used as the mobile phase, a demarcation at R_F 0.75 appears after charring. This may have been the limit of water migration, and such an effect may have enhanced the efficiency of the chromatographic process.

Fig. 3 shows the effect of the content of acetonitrile in the mobile phase on the R_F values of 10 sugars. All of the sugars were optimally resolved by using 7:3

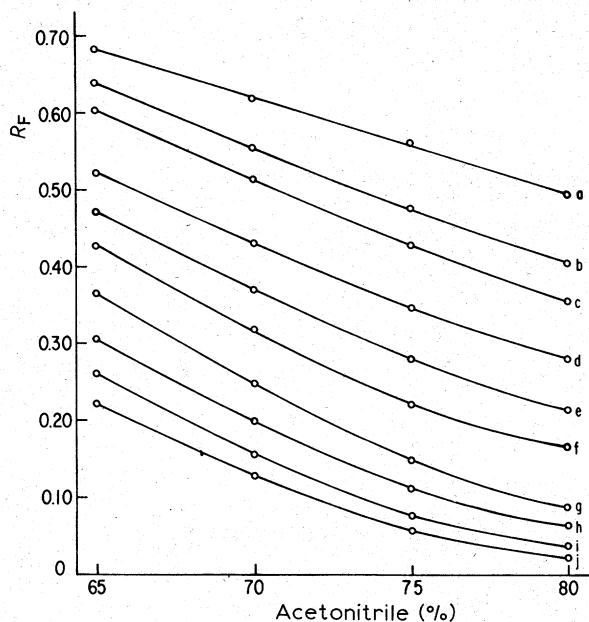


Fig. 3. Effect of the percentage of acetonitrile in the mobile phase on the R_F values of the following sugars and sugar derivatives: a, methyl β -D-arabinopyranoside; b, methyl α -D-mannopyranoside; c, methyl β -D-glucopyranoside; d, psicose; e, fructose; f, glucose; g, sucrose; h, maltose; i, melezitose; and j, raffinose.

TABLE III

R_F VALUES OF D-GLUCOSE DERIVATIVES IN 9:1 ACETONITRILE–WATER

D-Glucose derivative	R_F
1,2- <i>O</i> -Isopropylidene- α -D-glucofuranose	0.506
1,2:5,6-Di- <i>O</i> -isopropylidene- α -D-glucofuranose	0.869
3- <i>O</i> -Methyl-D-glucose	0.641
2-Acetamido-2-deoxy-D-glucose	0.092
4,6- <i>O</i> -Benzylidene-D-glucopyranose	0.632

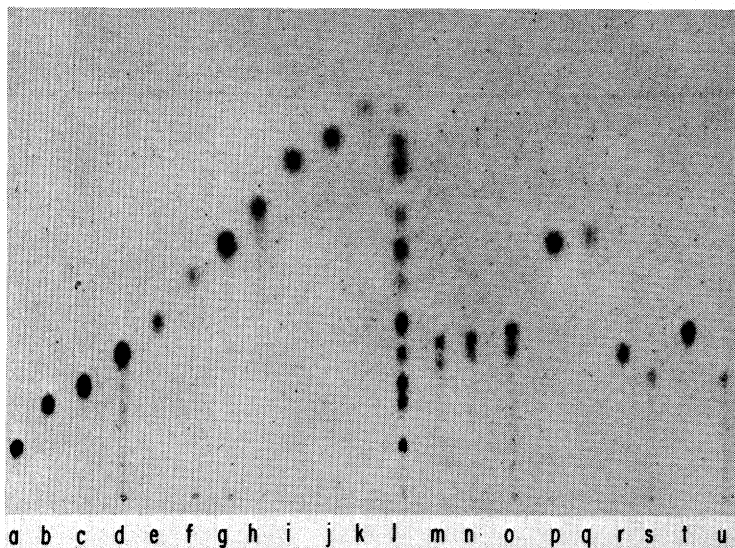


Fig. 4. Thin-layer chromatogram of some sugars and sugar mixtures on aminopropyl-bonded-phase plates, using 7:3 acetonitrile–water as the mobile phase. [Key: a, stachyose; b, raffinose; c, melezitose; d, maltose; e, sucrose; f, glucose; g, fructose; h, psicose; i, methyl β -D-glucopyranoside; j, methyl α -D-mannopyranoside; k, methyl β -D-arabinopyranoside; l, mixture of a–k; m, lactose plus lactulose; n, maltose plus maltulose; o, cellobiose plus cellobiulose; p, sorbose; q, tagatose; r, α,α -trehalose; s, gentiobiose; t, turanose; u, melibiose. In each case for the disaccharide pairs m–o, the ketodisaccharide has the higher R_F value].

acetonitrile–water as the mobile phase. Closely related glycosides (b,c), ketohexoses (d,e), and trisaccharides (i,j) are represented. Using this solvent system, we have determined the R_F values for mono-, di-, tri-, and tetra-saccharides, and these are listed in Table II. A chromatogram of some of these sugars is given in Fig. 4. The spots are more compact than is generally seen in the t.l.c. of sugars, and some closely related sugars are resolved. For example, the ketodisaccharides lactulose, maltulose, and cellobiulose are separated from their respective precursors lactose, maltose, and cellobiose. For sugar derivatives in which one (or more) of the hydroxyl groups is substituted, a less polar solvent-system (9:1 acetonitrile–water) is preferred. In Table III are listed the R_F values of some glucose derivatives, and Fig. 5 is a chromatogram showing their separation.

The aminopropyl-bonded-phase plates lessen the analysis time from that normally encountered in t.l.c. of sugars. Using 7:3 acetonitrile–water as the mobile phase, irrigation of the plate to a height of 10 cm takes ~ 22 min on these plates, compared with 25 min on a t.l.c. plate of underivatized silica. A more dramatic comparison is of the bonded-phase plate with an underivatized silica t.l.c. plate irrigated with 3:1:1 butanone–acetic acid–water, a solvent system traditionally used for the separation of sugars. This system typically requires an irrigation time of ~ 54 min for the front to reach a level of 10 cm. In Fig. 6 are plotted the values of the

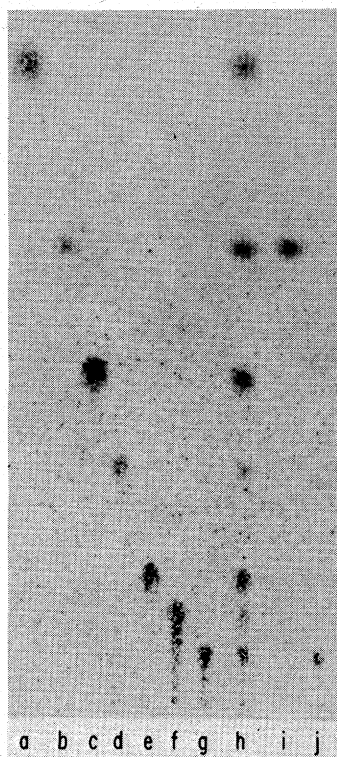


Fig. 5. Thin-layer chromatogram of some glucose derivatives on aminopropyl-bonded-phase plates, using 9:1 acetonitrile-water as the mobile phase. [Key: a, 1,2:5,6-di-*O*-isopropylidene- α -D-glucofuranose; b, 3-*O*-methyl-D-glucose; c, 1,2-*O*-isopropylidene- α -D-glucofuranose; d, methyl β -D-arabinopyranoside; e, methyl α -D-mannopyranoside; f, psicose; g, fructose; h, mixture of a-g; i, 4,6-*O*-benzylidene-D-glucopyranose; and j, 2-acetamido-2-deoxy-D-glucose.]

average linear velocity of the solvent vs distance of solvent development for these three chromatographic systems.

We have found that several sugars and sugar derivatives display no mobility on the aminopropyl-bonded-phase plates. These are listed in Table IV; these sugars apparently react at the origin after the sugars are spotted. It has been suggested^{11,12} that, in l.c. on aminopropyl-bonded-phase columns, significant formation of Schiff bases can occur between the amino and carbonyl groups in the sample. It has been advised¹¹ that such carbonyl compounds as ketosteroids or strongly reducing sugars not be used on such columns. On-column reactivity that is >10% has been observed¹¹ in l.c. of the aldopentoses, and glucose, galactose, and mannose. At a flow rate of 1.0 mL/min, 60% of injected ribose reacted. As expected, there is a strong correlation between the reactivity of a sugar and the percentage of the acyclic (free aldehyde) form in solution. Polarographic studies¹³ that examined the composition of free aldehyde of eight sugars at pH 7.0 in 0.1M aqueous solution gave the following results: ribose (8.5), allose (1.10), arabinose (0.22),

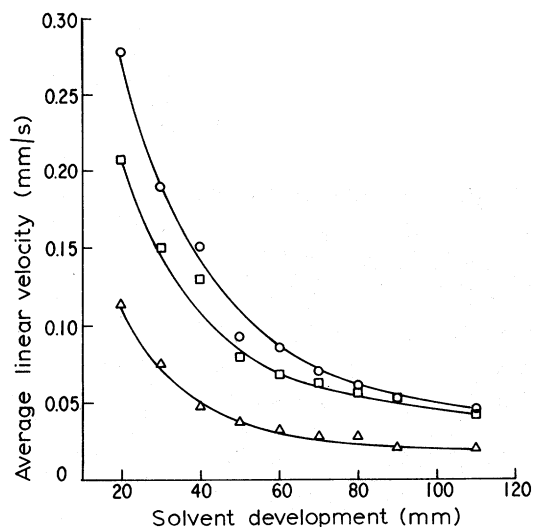


Fig. 6. Relationship between the average, linear velocity of the solvent front and the solvent-development distance for: \circ , aminopropyl-bonded-phase plates; and \square and \triangle , silica-gel plates. [In profiles represented by \circ and \square , plates were irrigated 7:3 with acetonitrile-water. The plate represented by \triangle was eluted with 3:1:1 butanone-acetic acid-water.]

lyxose (0.18), xylose (0.13), galactose (0.085), mannose (0.062), and glucose (0.022%). It was postulated¹³ that stabilizing effects of the terminal CH_2OH groups in hexopyranoses stabilize this form relative to the pentoses. Recently, l.c. conditions have been established¹⁴ to overcome the reactivity of the aldehydic sugar with the amino groups on the silica. This was accomplished by using a mobile phase consisting of acetonitrile and a phosphate buffer of pH 5.9. Lowering the pH in the environment of the aminopropyl groups from ~ 9.5 to 5.9 allowed xylose, arabinose, and glucose to be determined without on-column loss.

The sugars found in the present study to display no migration on aminopropyl-bonded-phase plates are those that cause difficulty in l.c. without buffered,

TABLE IV

ALDOSES AND ALDOSE DERIVATIVES HAVING NO MOBILITY ON (3-AMINOPROPYL)-SILICA BONDED-PHASE T.L.C. PLATES

<i>Tetrose and pentoses</i>	<i>Hexoses</i>	<i>Deoxy sugars</i>	<i>Sugar derivatives</i>
Erythrose	Galactose	2-Deoxy-D-erythro-pentose	2-Deoxy-2-(methylamino)-D-glucose
Ribose	Mannose	2-Deoxy-D-arabino-hexose	4,6-O-Ethylidene-D-glucopyranose
Arabinose	Talose	Rhamnose	
Xylose		Fucose	
Lyxose			

mobile phases. These are the reducing sugars having $>0.05\%$ of the acyclic form in aqueous solution. That a covalent linkage can be formed between some sugars and the amino groups on the plate was demonstrated by immersing a plate in a 1.0% solution of xylose in methanol for 20 h. After scraping off the gel, and repeatedly washing to remove the excess of xylose and methanol, the following microanalytical results were obtained: ash, 83.94, carbon, 7.78, and nitrogen, 1.67%. This xylosylated plate was yellow colored and was prepared from a derivatized plate that had been immersed in 3-APTS-hexane for 60 min (see Table I). From the increase in carbon content from 5.66 (before xylose reaction) to 7.78%, it was calculated that about half of the amino groups were derivatized with xylose. Similar results were obtained with rhamnose. Some reducing sugars migrate quite well and give pronounced spots, but apparently react to some extent at the origin and also as they move up the plate. This phenomenon is apparent in Fig. 4 for d, maltose; f, glucose; g, fructose, and h, psicose. In Fig. 5, streaking is apparent for f, psicose; and g, fructose. Only nonreducing sugars are devoid of tailing.

Attempts at rendering mobile the sugars listed in Table IV by incorporating NaH_2PO_4 in the mobile phase, or in the sugar solution applied to the plate, or both, have not been successful. Such precautions as pH adjustment that are applicable to 14 l.c. are not transferable to t.l.c., presumably because of the increased concentration of the sugar in the mixtures applied, especially when the plate is dried before it is placed in the developing tank. Preliminary results suggest that impregnating plates by immersing in 0.1M NaH_2PO_4 solution and then drying them, or by coating plates with aminopropyl-bonded-phase silica slurried in aqueous NaH_2PO_4 will prove useful. Important separations have been accomplished on the analogous, l.c. aminopropyl-silica columns^{15,16}, and improved approaches to resolution by t.l.c. will prove useful. During the course of this study, amino bonded-phase, "high-performance", t.l.c. plates have become commercially available (EM Science, Gibbstown, NJ). The usual advantages of having the capability to prepare one's own plates apply; these include versatility in varying the plate size, the degree of aminopropyl derivatization, and the thickness of the layer for preparative t.l.c.

EXPERIMENTAL

Plate derivatization. — T.l.c. plates precoated with silica gel were supplied by Alltech Associates, Inc., Deerfield, IL [catalog No. 16320 (20×20 cm) and catalog 16321 (5×20 cm)]. The plates were coated to a thickness of $250 \mu\text{m}$ and included 10% of calcium sulfate as a binder. (3-Aminopropyl)triethoxysilane (3-APTS) (Aldrich Chem. Co., Milwaukee, WI) was stored at 6° , and hexane was dried over calcium chloride. Derivatization of plates was conducted in standard, t.l.c. development tanks after adding dry hexane to a depth of 20 cm and stirring in the appropriate quantity (determined to be 1.0%, v/v) of 3-APTS. Two 20×20 -cm or eight 5×20 -cm plates were simultaneously derivatized for the optimal time of 15

min. Plates were then withdrawn, placed horizontally in a tray containing dry hexane, and kept for 15 min in order to wash out the excess of 3-APTS. After being dried *in vacuo* for 15 min at 60°, the plates were ready for use. No special precautions were needed in order to ensure that the plates were moisture-free, either before or after derivatization. It was possible to derivatize at least 12 large or 48 small plates successively, using the same immersing medium.

Plate characterization. — Analyses for carbon, nitrogen, and ash were made by Microanalysis, Inc. Wilmington, DE, and from these data, the degree of aminopropyl substitution onto silica and the C:N ratio in the bonded phase were calculated.

T.l.c. of sugars on aminopropyl-bonded-phase plates. — Lactulose, maltulose, and cellobiulose were kindly provided by Dr. K. B. Hicks, and laminarabiose by Dr. E. T. Reese; kojibiose was purchased from Koch-Light Laboratories, Ltd. (Colnbrook, England). Other sugars were obtained from Sigma (St. Louis, MO) and Aldrich (Milwaukee, WI) Chemical Companies. Aqueous solutions (20%, containing 10 p.p.m. of sodium azide) of sugars were prepared, and 0.2 μ L (4 μ g) was spotted at the plate origin. After irrigating the plates with acetonitrile–water, sugars were visibilized by spraying with 6:4:3 *tert*-butanol–ethanol–sulfuric acid, and heating in an oven for 5 min at 180°. The aminopropyl groups on the plate interfered with detection by several of the traditional, spray reagents for sugars. In many cases, the background gave colors more intense than those of the sugars being detected with the reagent.

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